



Effect of antiestrogens and aromatase inhibitor on basal growth of the human breast cancer cell line MCF-7 in serum-free medium

Janne Jensen^a, Jason W. Kitlen^a, Per Briand^a, Fernand Labrie^b, Anne E. Lykkesfeldt^{a,*}

^a Department of Tumor Endocrinology, Institute of Cancer Biology, Danish Cancer Society, Strandboulevarden 49, DK-2100 Copenhagen, Denmark

^b Laboratory of Molecular Endocrinology, CHUL Research Center, 2705 Boul. Laurier, Que., Canada G1V 4G2

Received 3 April 2002; accepted 3 December 2002

Abstract

Antiestrogens are efficient inhibitors of estrogen-mediated growth of human breast cancer. Besides inhibiting estradiol-stimulated growth, antiestrogens may have a direct growth-inhibitory effect on estrogen receptor (ER) positive cells and thus be more efficient than aromatase inhibitors, which will only abrogate estrogen-dependent tumor growth. To address this issue, we have used the human breast cancer cell line MCF-7/S9 as a model system which is maintained in a chemically defined medium without serum and estrogen. The addition of estradiol results in an increase in cell growth rate. Thus, the MCF-7/S9 cell line is estrogen-responsive but not estrogen-dependent. Three different types of antiestrogens, namely tamoxifen, ICI 182,780 and EM-652 were found to exert a significant and dose-dependent inhibition of basal growth of MCF-7/S9 cells. The growth-inhibitory effect of the three antiestrogens was prevented by simultaneous estradiol treatment. Antiestrogen treatment also reduced the basal pS2 mRNA expression level, thus indicating spontaneous estrogenic activity in the cells. However, treatment with the aromatase inhibitor had no effect on basal cell growth, excluding that endogenous estrogen synthesis is involved in basal growth. These data demonstrate that in addition to their estrogen antagonistic effect, antiestrogens have a direct growth-inhibitory effect which is ER-mediated. Consequently, in the subset of ER positive breast cancer patients with estrogen-independent tumor growth, antiestrogen therapy may be superior to treatment with aromatase inhibitors which only inhibit estrogen formation but do not affect cancer cell growth in the absence of estrogens.

© 2003 Elsevier Science Ltd. All rights reserved.

Keywords: Breast cancer; Growth inhibition; Antiestrogens; ICI 182,780; EM-652; Tamoxifen; Aromatase inhibitor; 4-OH-androstenedione; Estrogen receptor α

1. Introduction

A large proportion of breast cancer patients benefit from endocrine therapy and, for several decades, most of these patients have been treated with the antiestrogen tamoxifen as first line endocrine therapy. Tamoxifen acts by antagonizing the binding of estrogen to the ER in the breast cancer cells [1–3]. Accordingly, the patients who respond to tamoxifen therapy have ER positive breast tumors [4,5]. In post-menopausal women, inhibition of the growth of estrogen-dependent breast tumors may also be achieved by inhibiting endogenous estrogen synthesis via treatment with aromatase inhibitors [6,7]. During the last years, new second and third generation aromatase inhibitors have been developed and these compounds suppress in situ estrogen synthesis, endogenous estrogen levels in the tumor tissues as well as the plasma estrogen levels [8–11]. The third gen-

eration oral aromatase inhibitors—letrozole, anastrozole, vorozole and exemestane, are now considered established second-line hormonal agents for post-menopausal breast cancer patients [12]. Two recent clinical trials have revealed data demonstrating superiority of letrozole and anastrozole to tamoxifen as first line endocrine therapy [13,14].

Theoretically, treatments with an antiestrogen and an aromatase inhibitor may be equally efficient on growth of estrogen responsive tumors, provided complete estrogen deprivation can be achieved. However, data from experimental systems have shown that antiestrogens, besides being estrogen antagonists and blockers of estrogen action, may also have a direct growth-inhibitory effect in the absence of estrogens [15–19]. Such data indicate that antiestrogens may be potentially more efficient than aromatase inhibitors which only will suppress the estrogen-dependent tumor growth. In order to elucidate this hypothesis, we have used a model system with a subline of the ER positive human breast cancer cell line MCF-7, MCF-7/S9, which is cultivated in serum-free chemically defined medium in the

* Corresponding author. Tel.: +45-3525-7323; fax: +45-3525-7721.
E-mail address: al@cancer.dk (A.E. Lykkesfeldt).

absence estradiol [20]. In the present study, we have used the MCF-7/S9 cell line to analyze the effect of estradiol and three different types of antiestrogens, namely tamoxifen (triphenylethylene structure), ICI 182,780 (7 α -substituted estradiol compound) and EM-652 (benzopyran structure) on tumor cell growth. The expression levels of the estrogen-regulated genes: pS2, cathepsin D, α_1 -antitrypsin and c-erbB-2 have been determined in cultures grown in basal medium, in medium supplemented with either estradiol, estradiol + antiestrogen, or antiestrogen alone. Finally, growth studies with the second generation aromatase inhibitor 4-OH-androstenedione have been performed to elucidate whether an aromatase inhibitor inhibits basal growth of MCF-7/S9 cells.

2. Materials and methods

2.1. Cell lines and culture conditions

The MCF-7 cell line has been obtained from the Breast Cancer Task Force Cell Culture Bank, Mason Research Institute (Worcester, MA, USA). MCF-7/S9 has been established by gradually adapting MCF-7 cells to grow at a low serum concentration [21] before transfer to serum-free medium [20]. Since passage no. 387, the cell line has been propagated in phenol red-free DMEM/F12 (1:1) (Life Technologies, Roskilde, Denmark) without addition of serum or any growth factor (standard medium for MCF-7/S9), in T-flasks (Nunc, Roskilde, Denmark) coated with collagen (Cellon Bovine Dermal Collagen, In Vitro A/S, Fredensborg, Denmark). The MCF-7/S9 was subcultured once a week with a split ratio of approximately 1:10 and growth medium was changed every second or third day. Cells in passages 463–483 have been used in this study. In most experiments, 1 mg/ml BSA (Sigma–Aldrich A4919, St. Louis, MO) and 10 μ g/ml transferrin (Sigma–Aldrich) were added to stabilize the medium against endotoxins. The cell line is ER and progesterone receptor positive [20]. The HMT-3522/T4-2 cell line is a spontaneously transformed cell line originating from the HMT-3522 epithelial cell line derived from a benign breast tumor [22,23]. HMT-3522/T4-2 cells are ER negative breast cancer cells grown in serum-free medium consisting of DME/F12 (1:1) medium supplemented with 250 ng/ml insulin (Novo-Nordisk, Copenhagen, Denmark), 10 μ g/ml transferrin (Sigma–Aldrich), 2.6 ng/ml sodium selenite (Collaborative Research, Waltham, MA), 0.1 nM estradiol (Collaborative Research), 1 μ M hydrocortisone (Collaborative Research) and 5 μ g/ml sheep prolactin (Sigma–Aldrich).

2.2. Growth experiments

Cells were seeded in multi-well dishes with (1–2) \times 10⁴ cells per well (2 cm² wells, Nunc) in standard medium or standard medium with 1 mg/ml BSA and 10 μ g/ml

transferrin. Two days after seeding (day 0), experimental media were added and renewed every second or third day. Cell numbers were determined at the indicated days by trypsinating the cells followed by counting in a Bürker–Türk chamber. Four wells were used for each cell number determination. Stock solutions of 5 \times 10^{–3} M EM-652 (Laboratory of Molecular Endocrinology, CHUL Research Center, Que., Canada), 10^{–2} M estradiol (Collaborative Research), 10^{–3} M tamoxifen (AstraZeneca, Macclesfield, UK), 10^{–4} M ICI 182,780 (AstraZeneca), 10^{–4} M androstenedione (Sigma–Aldrich), 10^{–4} M testosterone (Sigma–Aldrich), 10^{–4} M 4-OH-androstenedione (Sigma–Aldrich) were dissolved in 96% ethanol and stored at –20 °C (estradiol and tamoxifen) or 4 °C. Newborn calf serum (NCS) was supplied by Gibco BRL.

2.3. Western analysis

The MCF-7/S9 cells were cultured in T25 flasks (Nunc) in standard medium supplemented with 1 mg/ml BSA and 10 μ g/ml transferrin. After treatment with either 10^{–10} M estradiol, 10^{–7} M ICI 182,780 or 10^{–7} M EM-652, the nearly confluent cultures were harvested using 400 μ l RIPA buffer (100 mM sodium chloride, 20 mM Trizma-base, 1% Triton X-100, 0.5% sodium desoxycholate, 0.1% SDS and 1 mM sodium–EDTA, pH 8.0). About 15 μ g of total protein per sample were run on 15% SDS-PAGE gels under reducing conditions. Proteins were transferred to an Immobilon-P membrane (Millipore, Bedford, MA, USA) by electroblotting. Immunostaining was carried out using a primary monoclonal mouse anti-human ER α antibody (1D5, DAKO, Glostrup, Denmark), a primary monoclonal mouse anti-human keratin 7 (Jiri Bartek, Danish Cancer Society, Denmark) and a secondary polyclonal rabbit anti-mouse IgG peroxidase conjugated antibody (P260, DAKO). The immunocomplex was visualized using the enhanced chemiluminescence (ECL) detection system (Amersham Pharmacia Biotech, Hørsholm, Denmark).

2.4. Northern analysis

The MCF-7/S9 cells were cultured in T75 flasks (Nunc) in standard medium supplemented with 1 mg/ml BSA and 10 μ g/ml transferrin. Cell cultures were treated for 2–3 days with estradiol, EM-652, ICI 182,780, or antiestrogen and estradiol in combination. The nearly confluent cultures were harvested with Trizol Reagent (Life Technologies) and total RNA was isolated from the lysates as recommended by the supplier. Poly(A)⁺RNA was isolated using Oligo(dT)₂₅ coupled magnetic beads (Dyna, Oslo, Norway) according to the manufacturer's manual. This procedure is scaled to give 2 μ g of poly(A)⁺RNA when 75 μ g total RNA starting material is used. The poly(A)⁺RNA was denatured by glyoxal–DMSO solution, separated on a 1% agarose gel and transferred to a nylon membrane (NY 13 N, Schleicher & Schuell, Dassel, Germany).

Plasmid probes were randomly labeled with [α - 32 P]dCTP (Amersham Pharmacia Biotech) using the Megaprime DNA labeling system (Amersham Pharmacia Biotech). The oligo probe to cathepsin D was end-labeled with [γ - 32 P]ATP (Amersham Pharmacia Biotech) by T4 polynucleotide kinase (Gibco BRL). Labeled probes were separated from unincorporated 32 P-labeled nucleotides by a NICK[®] Spin Column (Amersham Pharmacia Biotech) according to the manufacturer's manual. The blots were hybridized in RapidHyb hybridization solution (Amersham Pharmacia Biotech) containing 1.25×10^6 cpm probe/ml, and exposed to a PhosphorImager screen before quantification using the ImageQuant software (Molecular Dynamics (Amersham Pharmacia Biotech)). Before re-hybridizing a blot, the membrane was stripped in boiling water. Probes were: pS2: 0.6 kb PvuI to AseI fragment from the MCF-7 [24]; Cathepsin D, oligo, 35' mer: 5' TTA ACG TAG GTG CTG GAC TTG TCG CTG TTG TAC TT 3' (DNA Technology Aps, Aarhus, Denmark); α_1 -antitrypsin: 1.4 kb EcoRI fragment from a human liver cDNA library was cloned into the pBR322 vector [25]; ErbB-2: 0.9 kb AccI to BamHI fragment from pMAC117 [26]; 36B4: 1.1 kb PstI fragment from the MCF-7 cell line [27].

3. Results

3.1. MCF-7/S9 cells: ER α expression and binding characteristics

Western analysis has shown a 66 kDa protein with immunoreactivity towards the ER α specific antibody 1D5.

Ligand binding assay with 3 H-estradiol and Schatchard plot analysis of the data revealed specific estradiol binding sites corresponding to 340 fmol/mg cytosol protein and a K_D -value of 0.3×10^{-10} M. These analyses demonstrate that MCF-7/S9 cells express wild type ER α protein with normal estradiol binding characteristics.

3.2. Effect of estradiol on growth of MCF-7/S9 cells

Growth studies of cells in serum-free chemically defined medium with and without estradiol addition are shown in Fig. 1. MCF-7/S9 cells cultured in standard medium with 1 mg/ml BSA and 10 μ g/ml transferrin grow exponentially with a doubling time of about 44 h. In medium with 10^{-9} M estradiol added, the doubling time is reduced to 34 h, thus clearly demonstrating that this cell line has maintained the ability to respond to estradiol with increased growth. This cell line can, therefore, be classified as an estrogen-responsive but not estrogen-dependent cell line.

3.3. Growth inhibition with different types of antiestrogens

Dose-response experiments were performed with three different types of antiestrogens, namely ICI 182,780 which is a steroidal antiestrogen with a large side chain in the 7 α position [28,29], the non-steroidal antiestrogen EM-652 having a benzopyran structure [30,31] and tamoxifen, a compound having a triphenylethylene structure [1,32]. Fig. 2 shows that ICI 182,780 and EM-652 are about equally effective with respect to inhibition of growth of MCF-7/S9 cells. The inhibitory concentration to achieve

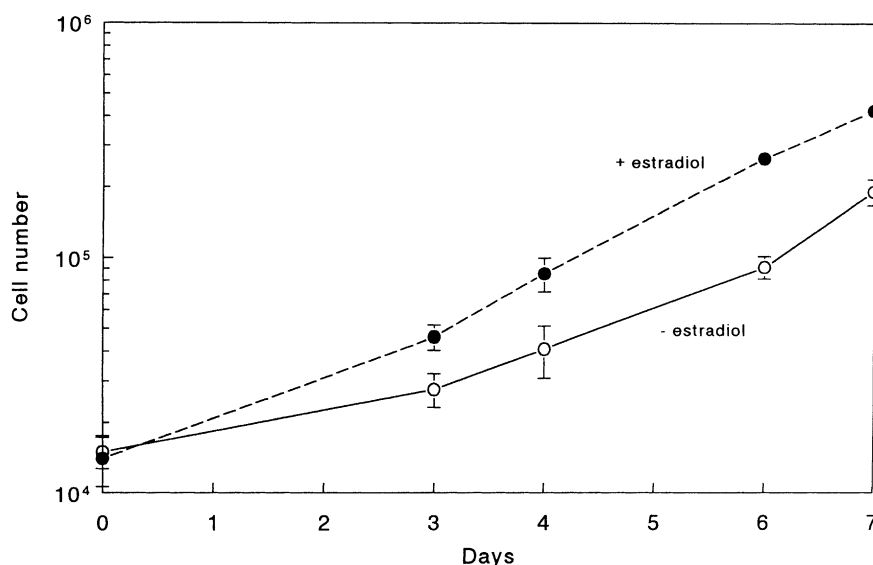


Fig. 1. Growth experiment with MCF-7/S9 grown in the absence and presence of estradiol. Cells were seeded in collagen coated multi-well dishes, 2×10^4 cells per well, in standard medium with 1 mg/ml BSA and 10 μ g/ml transferrin. Two days after seeding, medium supplemented with either no hormone or 10^{-9} M estradiol was added and renewed every second or third day. Cells were counted at days 0, 3, 4, 6 and 7 after the addition of estradiol. The error bars indicate standard deviations between four wells.

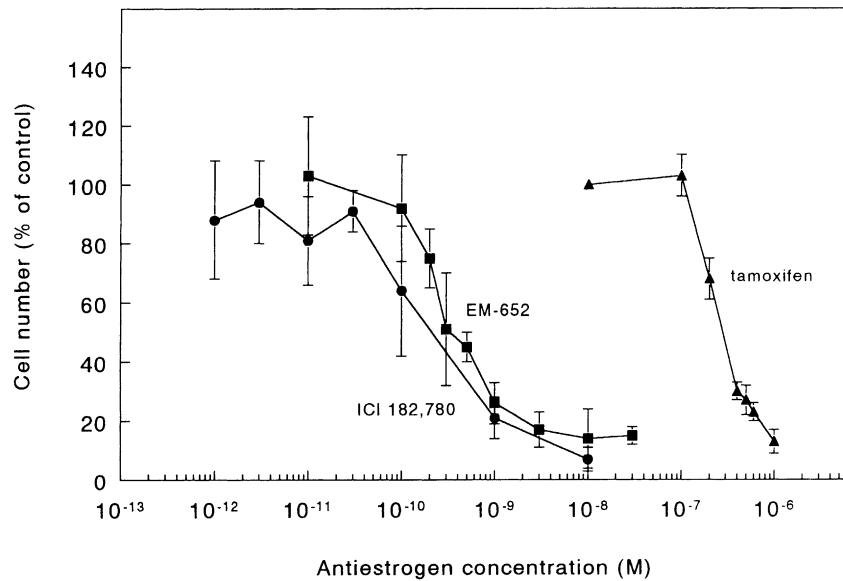


Fig. 2. Dose–response experiments with EM-652, ICI 182,780 or tamoxifen. MCF-7/S9 cells were seeded in multi-well dishes with $(1–2) \times 10^4$ cells per well in standard medium. Two days after seeding, medium containing the indicated concentrations of antiestrogen was added and renewed every second or third day. Cell numbers were determined 6 days after the addition of antiestrogen. In each experiment, an average of total cell number in four control wells not supplied with any hormone was defined as 100%. The mean values of three experiments are shown (expressed as percent of the corresponding control cultures). The error bars indicate standard deviation between the three experiments.

50% reduction in cell number was 2.1×10^{-10} M and 3.6×10^{-10} M for ICI 182,780 and EM-652, respectively. Tamoxifen also exerted a dose-dependent growth inhibition but the potency of this compound was about a 1000-fold lower, a tamoxifen concentration of 3×10^{-7} M resulting in 50% growth inhibition. For each antiestrogen, a maximal growth inhibition to approximately 10–20% of control could be achieved after 6 days of treatment. The growth inhibition obtained with 10^{-8} M ICI 182,780, 10^{-8} M EM-652 and 4×10^{-7} M tamoxifen could be completely abrogated by simultaneous addition of estradiol. No effect of antiestrogen treatment (10^{-8} M EM-652) was observed with the ER negative human breast cancer cell line HMT-3522/T4-2 (data not shown), strongly suggesting that the inhibitory effect of the antiestrogens is mediated via the ER.

3.4. Effect of ICI 182,780 and EM-652 on ER α protein stability

Western blot analyses were performed with lysates from MCF-7/S9 cells treated for different time intervals with ICI 182,780 or EM-652 to compare the stability of the receptor protein when bound to these compounds. ICI 182,780 treatment results in disappearance of ER α protein already after treatment for 2–3 h, whereas, EM-652 treatment only partially reduces the level of ER α protein (Fig. 3). This analysis demonstrates that the two very efficient antiestrogens have very different effects on the ER α protein level.

3.5. Effect of antiestrogens on the expression of estrogen-responsive genes

Estradiol regulates the expression of a large number of genes in human breast cancer cells. Based upon our knowledge from experiments performed with the parent

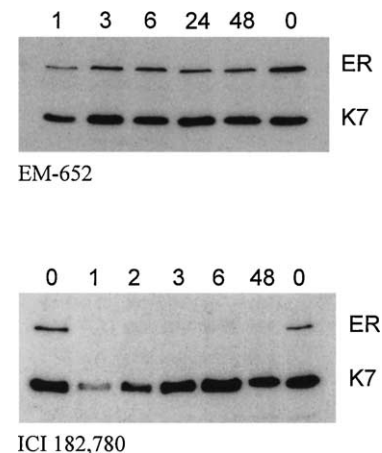


Fig. 3. Hormonal regulation of the ER α protein content in MCF-7/S9. Cells were grown in standard medium with 1 mg/ml BSA and 10 μ g/ml transferrin. After 0–48 h of treatment with either 10^{-7} M ICI 182,780 or 10^{-7} M EM-652, the nearly confluent cell cultures were harvested. ER α protein (M_w , 66 kDa) was detected by Western blot using a primary monoclonal mouse anti-human ER α antibody and a secondary polyclonal rabbit anti-mouse IgG peroxidase conjugated antibody, and was visualized by the enhanced chemiluminescence (ECL) detection system. Immunodetection of keratin 7 (K7; M_w , 40 kDa) was included as a control for loading.

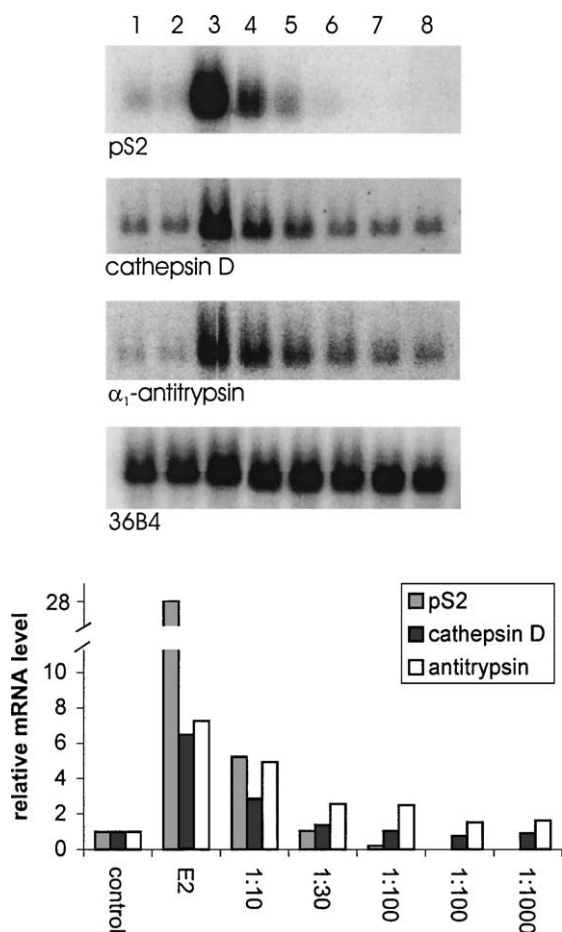


Fig. 4. Autoradiograms of Northern blots hybridized with ^{32}P -labeled probes for pS2, cathepsin D, α_1 -antitrypsin. The 2 μg of poly(A)⁺RNA from each condition were analyzed by hybridization of the same Northern blot with specific probes against mRNAs for pS2 (0.6kb), cathepsin D (2.2kb), α_1 -antitrypsin (1.4kb) and, as a control for RNA loading, 36B4 (1.2kb). Cells were treated for 3 days with different hormone concentrations. Lanes 1 and 2, controls, no hormone added; lane 3, 10^{-10} M estradiol; lane 4, 10^{-10} M estradiol + 10^{-9} M EM-652 (1:10); lane 5, 10^{-10} M estradiol + 3×10^{-9} M EM-652 (1:30); lane 6, 10^{-10} M estradiol + 10^{-8} M EM-652 (1:100); lane 7, 10^{-10} M estradiol + 10^{-8} M EM-652 (1:100); lane 8, 10^{-10} M estradiol + 10^{-7} M EM-652 (1:1000). (Below) Scan values relative to 36B4. Average of controls was defined as 1. The quantification was performed using ImageQuant software. One of the three experiments is shown, similar results were obtained in these experiments.

MCF-7 cell line grown in serum, we have chosen to study the expression of three genes upregulated by estradiol, namely pS2, cathepsin D and α_1 -antitrypsin, and one estradiol-downregulated gene, ErbB-2 [33–35]. In the MCF-7/S9 subline, a significant estradiol stimulation of pS2, cathepsin D and α_1 -antitrypsin was observed (Fig. 4). The highest estradiol induction was a 28-fold induction of pS2 mRNA levels. A significant increase of 6–7-fold was obtained with cathepsin D and α_1 -antitrypsin mRNA expression. Treatment of MCF-7/S9 cells with combinations of estradiol and the antiestrogen EM-652 reveals interesting differences with respect to expression of the

above-mentioned estrogen-responsive genes. The antiestrogen EM-652 downregulates the estradiol-induced expression of cathepsin D and α_1 -antitrypsin to about the expression level seen in control cultures (Fig. 4). Cultures treated with EM-652 alone expressed basal level of cathepsin D and α_1 -antitrypsin (data not shown). However, EM-652 is able to downregulate the expression of pS2 significantly below basal expression levels. A ratio of estradiol to EM-652 of 1:30 results in basal expression levels of pS2, whereas, further excess of EM-652 results in nearly undetectable levels of pS2 expression. Long term exposure of blots with RNA from control cultures and cultures treated with either EM-652 or ICI 182,780 alone demonstrated a faint band of pS2 in antiestrogen-treated cells corresponding to about 20% of the basal level (data not shown).

Estradiol significantly downregulates the ErbB-2 gene expression in MCF-7/S9 cells (Fig. 5). Addition of increasing

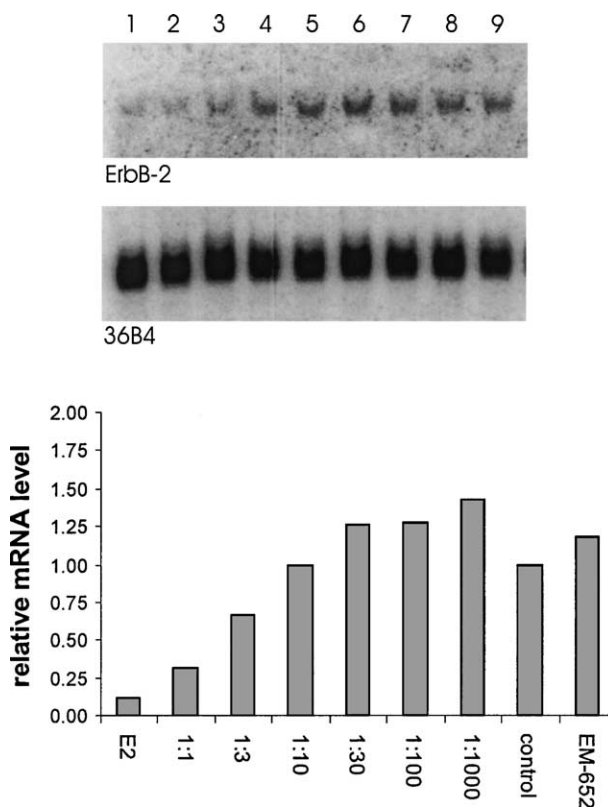


Fig. 5. Autoradiograms of Northern blot hybridized with ^{32}P -labeled probes for ErbB-2 and 36B4. The 2 μg of poly(A)⁺RNA from each condition were analyzed by hybridization of the same Northern blot with specific probes against ErbB-2 mRNA (4.5 kb) and 36B4 mRNA (1.2 kb) as a control for RNA loading. Cells were treated for 2 days with hormones as indicated. Lane 1, 10^{-10} M estradiol; lane 2, 10^{-10} M estradiol + 10^{-10} M EM-652; lane 3, 10^{-10} M estradiol + 3×10^{-10} M EM-652; lane 4, 10^{-10} M estradiol + 10^{-9} M EM-652; lane 5, 10^{-10} M estradiol + 3×10^{-9} M EM-652; lane 6, 10^{-10} M estradiol + 10^{-8} M EM-652; lane 7, 10^{-10} M estradiol + 10^{-7} M EM-652; lane 8, control, no hormone added; lane 9, 10^{-9} M EM-652. (Below) Scan values of ErbB-2 relative to 36B4. The control was defined as 1.00. The quantification was performed using ImageQuant software.

amounts of the antiestrogen EM-652 results in gradual abolishment of the estradiol-mediated downregulation. A ratio of estradiol to EM-652 of 1:10 results in an ErbB-2 expression corresponding to basal expression levels, whereas, higher EM-652 concentrations result in ErbB-2 levels a little higher than basal levels, similar to the cultures treated with EM-652 alone.

3.6. Effect of the aromatase inhibitor

4-OH-androstenedione on growth of MCF-7/S9 cells

Expression of pS2 is strongly correlated to estrogen-mediated growth [36,37]. The observation that MCF-7/S9 cells express pS2 when grown in basal medium without estradiol could suggest that these cells may have endogenous synthesis of active estrogens or that the estrogen receptor free of estrogen possesses significant basal activity. The growth-inhibitory effect of antiestrogens could be due to competition with low levels of endogenous estrogens. In order to test this hypothesis, several growth experiments with the aromatase inhibitor 4-OH-androstenedione have been performed. Fig. 6 shows that treatment with 10^{-7} M 4-OH-androstenedione for 6 days has no effect on the basal growth of MCF-7/S9. Increase of the 4-OH-androstenedione concentration to 10^{-6} M neither affected the growth of

MCF-7/S9 (data not shown). In addition, the androgens androstenedione and testosterone have no effect on cell growth. As expected, estradiol causes a significant increase in cell growth, and treatment with 10^{-8} M ICI 182,780 reduces growth to 25% of the control. This effect could be reversed by simultaneous addition of 10^{-8} M estradiol. The failure of the aromatase inhibitor to inhibit basal growth of MCF-7/S9 cells and the absence of growth stimulation with androstenedione or testosterone indicate that MCF-7/S9 cells in serum-free medium do not display aromatase activity.

Aromatase expression or activity in these cancer cells may require serum factors. In Fig. 7, growth experiments with MCF-7/S9 cells in basal medium with 10% newborn calf serum (NCS) are shown. NCS was chosen as the serum supplement since the level of active estrogen compounds in the NCS batch is extremely low (estradiol level below 40 pM), and a significant growth stimulation is observed by addition of 10^{-8} M estradiol. In this medium, a significant growth stimulation is also detected with androstenedione (10^{-8} M) and with testosterone (10^{-8} M), and this growth stimulation is abrogated in a dose-dependent manner by simultaneous addition of the aromatase inhibitor 4-OH-androstenedione. Complete inhibition of androgen-mediated growth was obtained with

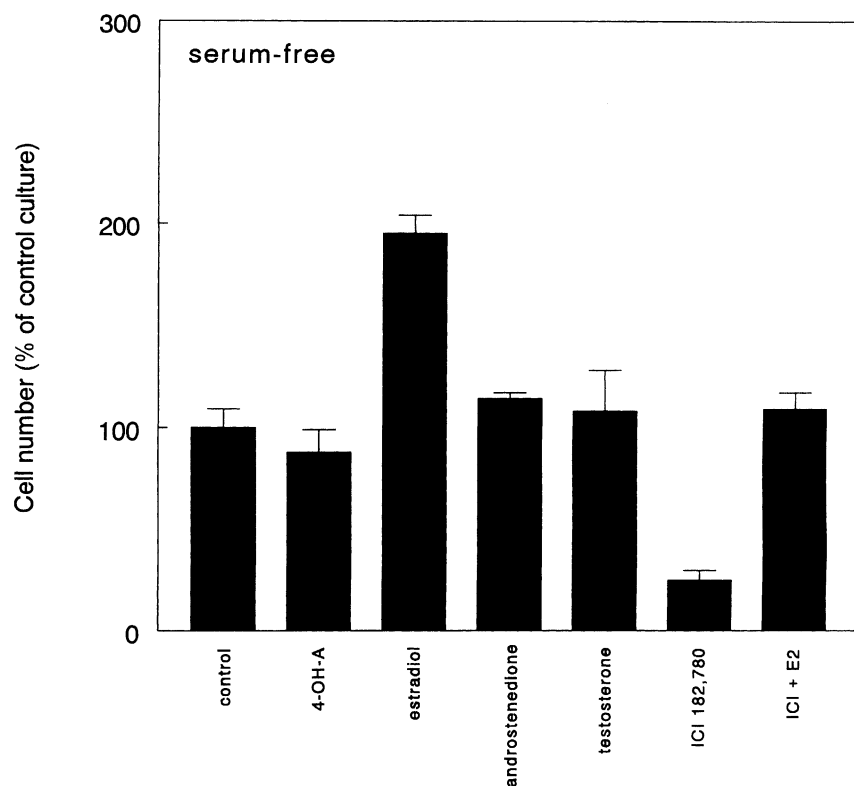


Fig. 6. Growth experiment in serum-free medium with aromatase inhibitor, estrogen, androgens, and antiestrogen. MCF-7/S9 cells were seeded in collagen coated multi-well dishes (2×10^4 cells per well) in standard medium with 1 mg/ml BSA and 10 μ g/ml transferrin. Two days after seeding, experimental medium with 10^{-7} M 4-OH-androstenedione (4-OH-A), 10^{-8} M estradiol, 10^{-8} M androstenedione, 10^{-8} M testosterone, 10^{-8} M ICI 182,780 or 10^{-8} M ICI 182,780 + 10^{-8} M estradiol (ICI + E2) was added. Medium was renewed every second or third day and cell number determined on day 6. The mean values of four wells (expressed as percent of the control culture) and the S.D.s are shown.

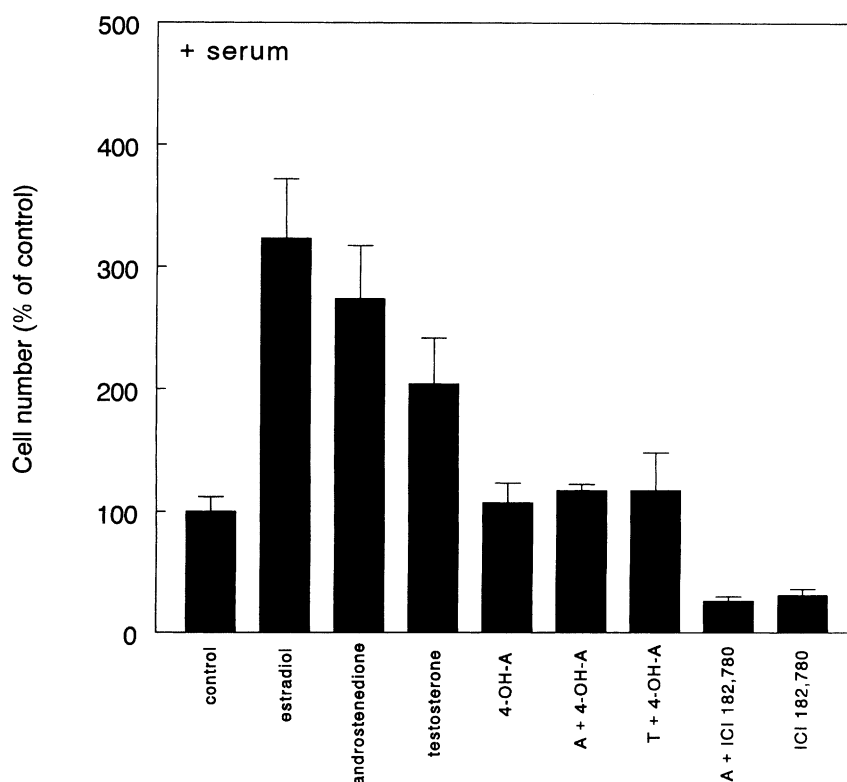


Fig. 7. Growth experiment in serum-containing medium supplemented with estrogen, androgens, aromatase inhibitor or antiestrogen. MCF-7/S9 cells were seeded in multiwell dishes (2×10^4 cells per well) in standard medium with 1 mg/ml BSA and 10 μ g/ml transferrin and 10% NCS. Two days after seeding, experimental medium with 10^{-8} M estradiol, 10^{-8} M androstenedione, 10^{-8} M testosterone, 10^{-7} M 4-OH-androstenedione (4-OH-A), 10^{-8} M androstenedione (A) + 10^{-7} M 4-OH-A, 10^{-8} M testosterone (T) + 10^{-7} M 4-OH-A, 10^{-8} M androstenedione (A) + 10^{-7} M ICI 182,780, or 10^{-7} M ICI 182,780 was added. Medium was renewed every second or third day and cell number determined on day 6. The mean values of four wells (expressed as percent of the control culture) and the S.D.s are shown.

10^{-7} M 4-OH-androstenedione (Fig. 7). Further increase in the 4-OH-androstenedione concentration did not reduce growth below control level (data not shown). The aromatase inhibitor has no effect when added alone in concentrations up to 10^{-6} M. ICI 182,780 (10^{-7} M) in combination with androstenedione (10^{-8} M) results in growth inhibition down to 20% of the control level like treatment with ICI 182,780 alone. These experiments demonstrate that MCF-7/S9 cells require serum factors to be growth-stimulated by androstenedione or testosterone. The aromatase inhibitor 4-OH-androstenedione totally abrogates androgen-mediated growth stimulation, whereas, the antiestrogen ICI 182,780 suppresses the androstenedione-mediated growth stimulation to a level significantly below the control level.

Preliminary data detecting aromatase mRNA by reverse transcription-polymerase chain reaction [38] disclosed a low amount of aromatase mRNA in MCF-7/S9 cells grown with 10% NCS, whereas, no aromatase mRNA was detected in MCF-7/S9 cells grown without serum. Aromatase enzyme activity was measured as $^3\text{H}_2\text{O}$ release in cultures grown with 1,2- ^3H androstenedione [39]. Significant $^3\text{H}_2\text{O}$ release was measured in cultures of MCF-7/S9 cells grown with 10% NCS and addition of 4-OH-androstenedione reduced the release of $^3\text{H}_2\text{O}$. These data support our sugges-

tion that MCF-7/S9 cells require serum factors to display aromatase activity.

4. Discussion

Several studies indicate that antiestrogens have direct growth-inhibitory effects in breast cancer cells [15–19]. However, most of these studies have been performed in medium with estrogen-depleted serum, or with cells transferred from serum-containing to serum-free medium. Therefore, it can not be totally excluded that small amounts of estrogenic compounds could influence basal growth in these systems. To avoid the problems with unknown factors supplied via the serum component, we have used a model system with the human breast cancer cell line MCF-7/S9 grown in serum-free chemically defined medium. This cell line has been passaged in serum-free medium for more than 100 passages and was subcultivated every week with a split ratio of approximately 1:10. The significant growth rate of MCF-7/S9 cells in basal medium is ideal for studies aimed at elucidating whether antiestrogens can exert growth inhibition on cells grown completely without estrogen supplementation. With this model system, we find

that three different types of antiestrogens, namely tamoxifen, ICI 182,780 and EM-652 exert a significant direct growth-inhibitory effect. Interestingly, the potency of ICI 182,780 and EM-652 was very similar and about a 1000-fold more efficient than tamoxifen. However, maximal growth inhibition was the same for all antiestrogens, namely a decrease to 10–20% of the cell number observed in the control culture. The MCF-7/S9 cell line expresses wild type ER α and the antiestrogen-mediated growth inhibition can be abolished by simultaneous addition of estrogen, thus indicating the involvement of ER.

The mechanisms of action of tamoxifen and ICI 182,780 are very different [40]. Tamoxifen stabilizes the ER protein [41] and inhibits growth, primarily by inhibiting the transcription via the AF-2 activity of the ER α . ICI 182,780 destabilizes the ER α protein [42,43] and the transcription is inhibited both due to the degradation of the receptor and due to the lack of coactivator recruitment to ICI 182,780 bound receptors. The new antiestrogen EM-652 is more potent than ICI 182,780 with respect to inhibition of estradiol-stimulated growth [30]. However, the mechanism of action appears to be somewhat different from ICI 182,780 as we find that EM-652 binding has very little influence on ER α stability in this system. This is in concert with the observation of others [44], although other studies have shown an EM-652 induced loss of ER α in the mammary gland and uterus of rats treated with EM-652 (Labrie, unpublished observations).

So far, we have found pure estrogen antagonistic activity of the EM-652 compound in the MCF-7/S9 cell line. EM-652 completely abolished the estrogen-induced expression of cathepsin D and α_1 -antitrypsin, and EM-652 alone had no effect on the expression of these genes. This is in contrast to tamoxifen which has agonistic activity with respect to the induction of both cathepsin D and α_1 -antitrypsin mRNA in MCF-7 cells [33]. The expression of cathepsin D and α_1 -antitrypsin is upregulated by estrogen. However, MCF-7 cells grown with serum have a constitutive expression level of these genes, as indicated by our previous finding that ICI 182,780 can not completely abolish the expression of cathepsin D and α_1 -antitrypsin [33]. MCF-7/S9 cells also appear to have a constitutive expression level of cathepsin D and α_1 -antitrypsin.

The pS2 gene is probably the most estrogen-sensitive gene [45], and expression of pS2 mRNA requires ER activity as ICI 182,780 treatment results in undetectable expression levels of the pS2 mRNA in MCF-7 cells grown with serum [33]. In the present study, a low basal pS2 mRNA level could be detected and estradiol treatment resulted in a 28-fold induction in the expression level. Treatment with EM-652 and also with ICI 182,780 suppressed the pS2 expression to a level significantly below the basal expression level. This observation indicates that a fraction of the ERs in MCF-7/S9 cells may be in a spontaneously activated state. The antiestrogen EM-652 acts as an estrogen antagonist on the expression of the estrogen downregulated ErbB-2 gene in MCF-7/S9 cells.

Activated ERs could indicate endogenous synthesis of estrogenic compounds by the MCF-7/S9 cells or ligand-independent activation via phosphorylation of the ER. Our studies with the second generation aromatase inhibitor 4-OH-androstenedione clearly demonstrate that this inhibitor is able to completely abrogate the androgen-mediated growth stimulation when MCF-7/S9 cells are grown with serum. However, the aromatase inhibitor has no effect on the growth of MCF-7/S9 cells in serum-free medium, thus indicating that basal growth does not involve endogenous estrogen synthesis. Lack of endogenous estrogen synthesis in MCF-7/S9 cells grown in basal medium is also indicated by the observation that addition of the aromatase enzyme substrates, androstenedione or testosterone, does not result in growth stimulation. Although the expression of pS2 in MCF-7/S9 cells grown in basal medium indicates ER activity in the cells, basal pS2 mRNA expression level is only 4% of the level found in cells which are growth-stimulated with estradiol. The lack of effect of an aromatase inhibitor on basal growth of MCF-7/S9 cells is, therefore, not surprising. These data do not exclude a low endogenous synthesis of estrogens in MCF-7/S9 cells, but the amount of estrogens is certainly not sufficient to influence growth significantly.

One could speculate whether the low pS2 expression observed in MCF-7/S9 cells grown in basal medium originates from exogenous estrogenic compounds supplied via the culture medium or from the plastic culture flasks. However, the medium is phenol red-free and the culture flasks do not contain plasticizers with known estrogenic activity like phthalates or bisphenols. Therefore, we find it unlikely that the pS2 expression arises from unknown estrogenic compounds in the culture medium. In patients, estrogenic activity may arise from natural compounds or xenoestrogens in the food. The activity of these exogenous estrogenic compounds may be suppressed by antiestrogen therapy but not by treatment with aromatase inhibitors.

The ability of the MCF-7/S9 cells to grow in serum-free chemically defined medium without estradiol or growth factor supplementation indicates that this cell line may be able to synthesize growth factors [46]. Therefore, it is not improbable that the ERs in MCF-7/S9 cells may be activated by phosphorylation mediated via growth factor signaling pathways as shown in other studies [47–53]. The observed pS2 expression in cells grown in basal medium may thus arise from activation of the pS2 transcription by unliganded ERs. Based on the calculation that the pS2 expression in cells grown in basal medium is only 4% of the level in estradiol-treated cells, the activity of the activated ERs may be insignificant for growth stimulation. Consequently, the significant growth inhibition obtained by antiestrogen treatment is probably not exclusively arising from abrogation of ligand-independent ER activity. Most likely, growth inhibition is a direct effect mediated via changes of conformation of the ER, which could induce the expression of inhibitory factors such as TGF- β or IGFBP-3 [54,55] or interfere with other signal transduction pathways [56,57]. Analysis of the

gene expression profiles in MCF-7/S9 cells grown in basal medium, medium with estradiol or with antiestrogen should be performed to identify the genes which may be involved in the direct growth inhibition.

This clear demonstration that three different antiestrogens exert a significant growth inhibition on the estrogen-independent growth of the MCF-7/S9 cell line, whereas, the potent aromatase inhibitor 4-OH-androstenedione has no effect on the basal cell growth suggests that antiestrogens under optimal conditions may be superior to aromatase inhibitors with respect to growth inhibition of a subset of ER positive human breast cancers. Our model system may reflect human breast tumors displaying ligand-independently activated ERs, tumors which are growth stimulated by exogenously derived estrogenic compounds or ER positive tumors growing estrogen-independently. Our observation could seem in contrast with the recent clinical findings that the new third-generation aromatase inhibitors letrozole, anastrozole, vorozole and exemestane show superiority compared with tamoxifen [12]. The mixed agonist antagonist action of tamoxifen may, however, explain the lower efficacy of this antiestrogen.

Acknowledgements

We thank Inger Heiberg for skillful technical assistance and Pia Riis Kofoed-Hansen for secretarial help. The pS2 probe was a gift from Professor P. Chambon. ICI 182,780 and tamoxifen were kindly provided from AstraZeneca. Antibody to cytokeratin K7 was supplied by Dr. Jiri Bartek. The project has been supported by grants from Danish Cancer Society.

References

- [1] R.I. Nicholson, J.S. Syne, C.P. Daniel, K. Griffiths, The binding of tamoxifen to estrogen receptor proteins under equilibrium and non-equilibrium conditions, *Eur. J. Cancer* 15 (1979) 317–329.
- [2] S. Bardon, F. Vignon, D. Derocq, H. Rochefort, The antiproliferative effect of tamoxifen in breast cancer cells: mediation by the estrogen receptor, *Mol. Cell. Endocrinol.* 35 (1984) 89–96.
- [3] R.R. Reddel, L.C. Murphy, R.E. Hall, R.L. Sutherland, Differential sensitivity of human breast cancer cell lines to the growth-inhibitory effects of tamoxifen, *Cancer Res.* 45 (1985) 1525–1531.
- [4] H. Mouridsen, T. Palshof, J. Patterson, L. Battersby, Tamoxifen in advanced breast cancer, *Cancer Treat. Rev.* 5 (1978) 131–141.
- [5] C.K. Osborne, M.G. Yochmowitz, W.A. Knight, W.L. McGuire, The value of estrogen and progesterone receptors in the treatment of breast cancer, *Cancer* 46 (1980) 2884–2888.
- [6] B.J. Roseman, A.U. Buzdar, S.E. Singletary, Use of aromatase inhibitors in post-menopausal women with advanced breast cancer, *J. Surg. Oncol.* 66 (1997) 215–220.
- [7] A.S. Bhatnagar, W.R. Miller, Pharmacology of inhibitors of estrogen biosynthesis, in: G.V.R. Born (Ed.), *Handbook of Experimental Pharmacology*, vol 135/2. Estrogens, Antiestrogens. Part II: Pharmacology and Clinical Application of Estrogens and Antiestrogens, in: M. Oettel, E. Schillinger (Eds.), Springer-Verlag, Heidelberg, Berlin, 1999, pp. 223–230.
- [8] J. Geisler, N. King, M. Dowsett, L. Ottestad, S. Lundgren, P. Walton, P.O. Kormeset, P.E. Lønning, Influence of anastrozole (Arimidex), a selective, non-steroidal aromatase inhibitor, on in vivo aromatization and plasma estrogen levels in post-menopausal women with breast cancer, *Br. J. Cancer* 74 (1996) 1286–1291.
- [9] I.E. Smith, A. Norton, Fadrozole and letrozole in advanced breast cancer: clinical and biochemical effects, *Breast Cancer Res. Treat.* 49 (1998) S67–S71.
- [10] W.R. Miller, J. Telford, C. Love, R.C.F. Leonard, S. Hillier, H. Gundacker, H. Smith, J.M. Dixon, Effects of letrozole as primary medical therapy on in situ estrogen synthesis and endogenous estrogen levels within the breast, *Breast* 7 (1998) 273–276.
- [11] C. Harper-Wynne, M. Dowsett, Recent advances in the clinical application of aromatase inhibitors, *J. Steroid Biochem. Mol. Biol.* 76 (2001) 179–186.
- [12] P.E. Goss, K. Strasser, Aromatase inhibitors in the treatment and prevention of breast cancer, *J. Clin. Oncol.* 19 (2001) 881–894.
- [13] H. Mouridsen, M. Gershanovich, Y. Sun, et al., Superior efficacy of letrozole versus tamoxifen as first-line therapy for post-menopausal women with advanced breast cancer: results of a phase III study of the international letrozole breast cancer group, *J. Clin. Oncol.* 19 (2001) 2595–2606.
- [14] A. Nabholz, A. Buzdar, M. Pollak, et al., Anastrozole is superior to tamoxifen as first-line therapy for advanced breast cancer in post-menopausal women: results of a North American multicenter randomized trial, *J. Clin. Oncol.* 18 (2000) 3758–3767.
- [15] J.C. Allegra, M.E. Lippman, The effects of 17 β estradiol and tamoxifen on the ZR-75-1 human breast cancer cell line in defined medium, *Eur. J. Cancer* 16 (1980) 1007–1015.
- [16] B.S. Katzenellenbogen, K.L. Kendra, M.J. Norman, Y. Berthois, Proliferation, hormonal responsiveness, and estrogen receptor content of MCF-7 human breast cancer cells grown in the short- and long-term absence of estrogens, *Cancer Res.* 47 (1987) 4355–4360.
- [17] F. Vignon, M.-M. Bouton, H. Rochefort, Antiestrogens inhibit the mitogenic effect of growth factors on breast cancer cells in the total absence of estrogens, *Biochem. Biophys. Res. Commun.* 146 (1987) 1502–1508.
- [18] G. Freiss, C. Puech, F. Vignon, Extinction of insulin-like growth factor-I mitogenic signaling by antiestrogen-stimulated Fas-associated protein tyrosine phosphatase-1 in human breast cancer cells, *Mol. Endocrinol.* 12 (1998) 568–579.
- [19] M. Salerno, D. Sisci, L. Mauro, M.A. Guvakova, S. Ando, E. Surmacz, Insulin receptor substrate 1 is a target for the pure antiestrogen ICI 182,780 in breast cancer cells, *Int. J. Cancer* 81 (1999) 299–304.
- [20] P. Briand, A.E. Lykkesfeldt, Long-term cultivation of a human breast cancer cell line, MCF-7, in a chemically defined medium: effect of estradiol, *Anticancer Res.* 6 (1986) 85–90.
- [21] P. Briand, A.E. Lykkesfeldt, Effect of estrogen and antiestrogen on the human breast cancer cell line MCF-7 adapted to growth at low serum concentration, *Cancer Res.* 44 (1984) 1114–1119.
- [22] P. Briand, O.W. Petersen, B. van Deurs, A new diploid non-tumorigenic human breast epithelial cell line isolated and propagated in chemically defined medium, *In Vitro Cell. Dev. Biol.* 23 (1987) 181–188.
- [23] P. Briand, K.V. Nielsen, M.W. Madsen, O.W. Petersen, Trisomy 7p and malignant transformation of human breast epithelial cells following epidermal growth factor withdrawal, *Cancer Res.* 56 (1996) 2039–2044.
- [24] S.B. Jakowlew, R. Breathnach, J.M. Jeltsch, P. Masiakowski, P. Chambon, Sequence of the pS2 mRNA induced by estrogen in the human breast cancer cell lines MCF-7, *Nucl. Acids Res.* 12 (1984) 2861–2878.
- [25] G.L. Long, T. Chandra, S.L. Woo, E.W. Davie, K. Jurachi, Complete sequence of the cDNA for human α_1 -antitrypsin and the gene for the S variant, *Biochemistry* 23 (1984) 4828–4837.

- [26] C.R. King, M.H. Kraus, S.A. Aaronson, Amplification of a novel v-erbB-related gene in a human mammary carcinoma, *Science* 229 (1985) 974–976.
- [27] J. Laborda, 36B4 cDNA used as an estradiol-independent mRNA control is the cDNA for human acidic ribosomal phosphoprotein PO, *Nucl. Acids Res.* 19 (1991) 3998.
- [28] A.E. Wakeling, M. Dukes, J. Bowler, A potent specific pure antiestrogen with clinical potential, *Cancer Res.* 51 (1991) 3867–3873.
- [29] A. Howell, D. DeFriend, J. Robertson, R. Blamey, P. Walton, Response to a specific antiestrogen (ICI 182,780) in tamoxifen-resistant breast cancer, *Lancet* 345 (1995) 29–30.
- [30] J. Simard, C. Labrie, A. Bélanger, S. Gauthier, S.M. Sigh, Y. Merand, F. Labrie, Characterization of the effects of the novel non-steroidal antiestrogen EM-800 on basal and estrogen-induced proliferation of T-47D, *Int. J. Cancer* 73 (1997) 104–112.
- [31] F. Labrie, C. Labrie, A. Bélanger, et al., Pure selective estrogen receptor modulators, new molecules having absolute cell specificity ranging from pure antiestrogenic to complete estrogen-like activities, in: E.M. Scolnick, F.M. Richards, D.S. Eisenberg, P.S. Kim (Eds.), *Advances in Protein Chemistry: Drug Discovery and Design*, vol. 56. Academic Press, San Diego, CA, 2001, pp. 293–368.
- [32] P. Daniel, S.J. Gaskell, H. Bishop, C. Chambell, R.I. Nicholson, Determination of tamoxifen and biologically active metabolites in human breast tumors and plasma, *Eur. J. Cancer. Clin. Oncol.* 17 (1981) 1183–1189.
- [33] A.E. Lykkesfeldt, M.W. Madsen, P. Briand, Altered expression of estrogen-regulated genes in a tamoxifen-resistant and ICI 164,384 and ICI 182,780 sensitive human breast cancer cell line, MCF-7/TAM^R-1, *Cancer Res.* 54 (1994) 1587–1595.
- [34] S.S. Larsen, M.W. Madsen, B.L. Jensen, A.E. Lykkesfeldt, Resistance of human breast-cancer cells to the pure steroidal anti-estrogen ICI 182,780 is not associated with a general loss of estrogen-receptor expression or lack of estrogen responsiveness, *Int. J. Cancer* 72 (1997) 1129–1136.
- [35] S.S. Larsen, M. Egeblad, M. Jäättelä, A.E. Lykkesfeldt, Acquired antiestrogen resistance in MCF-7 human breast cancer sublines is not accomplished by altered expression of receptors in the ErbB-family, *Breast Cancer Res. Treat.* 58 (1999) 41–56.
- [36] J.A. Foekens, M.C. Rio, P. Seguin, et al., Prediction of relapse and survival in breast cancer patients by pS2 protein status, *Cancer Res.* 50 (1990) 3832–3837.
- [37] S. Ribieras, C. Tomasetto, M.-C. Rio, The pS2/TFF1 trefoil factor, from basic research to clinical applications, *Biochim. Biophys. Acta* 1378 (1998) F61–F77.
- [38] R.D. Koos, P.K. Banks, S.E. Inkster, W. Yue, A.M.H. Brodie, Detection of aromatase and keratinocyte growth factor expression in breast tumors using reverse transcription-polymerase chain reaction, *J. Steroid. Biochem. Mol. Biol.* 45 (1993) 217–225.
- [39] E.A. Thompson, P.K. Siiteri, Utilization of oxygen and reduced nicotinamide adenine denucleotide phosphate by human placental microsomes during aromatization of androstenedione, *J. Biol. Chem.* 249 (1974) 5364–5372.
- [40] A. Howell, C.K. Osborne, C. Morris, A.E. Wakeling, ICI 182,780 (FaslodexTM): development of a novel, “pure” antiestrogen, *Cancer* 89 (2000) 817–825.
- [41] K.B. Horwitz, W.L. McGuire, Nuclear mechanisms of estrogen action: effects of estradiol and anti-estrogens on estrogen receptors and nuclear receptor processing, *J. Biol. Chem.* 253 (1978) 8185–8191.
- [42] S. Dauvois, P.S. Daniellian, R. White, M.G. Parker, Antiestrogen ICI 164,384 reduces cellular estrogen receptor content by increasing its turnover, *Proc. Natl. Acad. Sci. U.S.A.* 89 (1992) 4037–4041.
- [43] B.L. Jensen, J. Skouv, B.K. Lundholt, A.E. Lykkesfeldt, Differential regulation of specific genes in MCF-7 and the ICI 182,780-resistant cell line MCF-7/182^R-6, *Br. J. Cancer* 79 (1999) 386–392.
- [44] J.I. MacGregor Schafer, H. Liu, D.A. Tonetti, V.C. Jordan, The interaction of raloxifene and the active metabolite of the antiestrogen EM-800 (SC 5705) with the human estrogen receptor, *Cancer Res.* 59 (1999) 4308–4313.
- [45] P. Masiakowski, R. Breathnach, J. Bloch, F. Gannon, A. Krust, P. Chambon, Cloning of cDNA sequences of hormone-regulated genes from the MCF-7 human breast cancer cell line, *Nucl. Acids Res.* 10 (1982) 7895–7903.
- [46] I. Laursen, P. Briand, A.E. Lykkesfeldt, Serum albumin as a modulator on growth of the human breast cancer cell line, MCF-7, *Anticancer Res.* 10 (1990) 343–352.
- [47] D.M. Ignar-Trowbridge, K.G. Nelson, M.C. Bidwell, et al., Coupling of dual signaling pathways: epidermal growth factor action involves the estrogen receptor, *Proc. Natl. Acad. Sci. U.S.A.* 89 (1992) 4658–4662.
- [48] S.M. Aronica, B.S. Katzenellenbogen, Stimulation of ER-mediated transcription and alteration in the phosphorylation state of the rat uterine ER by estrogen, cyclic adenosine monophosphate, and insulin-like growth factor-I, *Mol. Endocrinol.* 7 (1993) 743–752.
- [49] S.F. Arnold, J.D. Obourn, H. Jaffe, A.C. Notides, Phosphorylation of the human ER by mitogen-activated protein kinase and casein kinase. Part II: Consequence on DNA binding, *J. Steroid Biochem. Mol. Biol.* 55 (1995) 163–172.
- [50] S. Kato, H. Endoh, Y. Masuhiro, et al., Activation of the estrogen receptor through phosphorylation by mitogen-activated protein kinase, *Science* 270 (1995) 1491–1494.
- [51] R.J. Pietras, J. Arboleda, D.M. Reese, et al., HER-2 tyrosine kinase pathway targets estrogen receptor and promotes hormone-independent growth in human breast cancer cells, *Oncogene* 10 (1995) 2435–2446.
- [52] G. Bunone, P.-A. Briand, R.J. Miksicek, D. Picard, Activation of the unliganded estrogen receptor by EGF involves the MAP kinase pathway and direct phosphorylation, *EMBO J.* 15 (1996) 2174–2183.
- [53] N.L. Weigel, Y. Zhang, Ligand-independent activation of steroid hormone receptors, *J. Mol. Med.* 76 (1998) 469–479.
- [54] C. Knabbe, M.E. Lippman, L.M. Wakefield, et al., Evidence that transforming growth factor- β is a hormonally regulated negative growth factor in human breast cancer cells, *Cell* 48 (1987) 417–428.
- [55] H. Huynh, X. Yang, M. Pollak, Estradiol and antiestrogens regulate a growth inhibitory insulin-like growth factor binding protein 3 autocrine loop in human breast cancer cells, *J. Biol. Chem.* 27 (1996) 1016–1021.
- [56] D. Chablos, A. Philips, H. Rochefort, Genomic cross-talk between the estrogen receptor and growth factor regulatory pathways in estrogen target tissues, *Sem. Cancer Biol.* 5 (1994) 361–368.
- [57] A. de Cupis, R.E. Favoni, Estrogen/growth factor cross-talk in breast carcinoma: a specific target for novel antiestrogens, *TIPS* 18 (1997) 245–251.